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The insulin receptor tyrosyl kinase phosphorylates holomeric forms of the guanine nucleotide regulatory proteins G_i and G_o

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An affinity purified human insulin receptor preparation was shown to phosphorylate the α- and β-subunits of the guanine nucleotide-regulatory proteins G_i and G_o, derived from bovine brain. The presence of insulin stimulated the rate of their phosphorylation some 2-fold. The presence of G_i and G_o did not affect the degree of autophosphorylation of the β-subunit of the insulin receptor. Under conditions known to cause the dissociation of G_i and G_o into their constituent subunits then phosphorylation of G_i and G_o by the insulin receptor was abolished. The α-subunits of G_i and G_o could be selectively phosphorylated by the insulin receptor tyrosyl kinase using appropriate concentrations of Mg^{2+} and GTP-γ-S.

G-protein; Guanine nucleotide; Insulin; Phosphorylation; Receptor; Kinase

1. INTRODUCTION

The receptors for insulin, EGF and PDGF as well as a variety of kinases expressed by oncogenes exhibit the ability to phosphorylate target proteins on tyrosine residues [1–3]. As yet there is no clear indication of the physiological role of such observations. However, it has been noted that cells which express mutant insulin receptors with defective tyrosyl kinase activity are unresponsive to insulin in terms of eliciting the stimulation of glucose uptake [4].

Many cell surface receptors appear to generate intracellular signals by modulating the activity of an appropriate effector system via distinct guanine nucleotide-regulatory proteins. Thus stimulation and inhibition of adenylate cyclase is mediated via G_s and G_i, respectively [5–8] and the *ras* gene product p21 is believed to couple receptors for certain growth factors to the stimulation of inositol phospholipid metabolism [9]. Other G-proteins have been identified, such as G_o [10,11] and $G_{\alpha 25}$ [12], whose function has yet to be determined.

It has been suggested (see [2,13,14]) that insulin can interact with the guanine nucleotide-regulatory protein system based upon its ability to inhibit adenylate cyclase [15] and activate specific species of cyclic AMP phosphodiesterase [16]. Such experiments indicated that insulin might interact with a novel G-protein called G_{ins}. This has tentatively been identified [17] as having a 25 kDa α-subunit like a recently characterised novel G-protein found in placenta and platelets [12]. As pertussis toxin, which causes the ADP-ribosylation of both G_i and G_o, prevents insulin activating the 'dense-vesicle' cyclic AMP phosphodiesterase in both liver and adipocytes it has been suggested that insulin might

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also elicit certain effects by interacting with G_i [18,19].

Here we demonstrate that the purified insulin receptor can cause the phosphorylation of the holomeric forms of G_i and G_o purified from bovine brain.

2. MATERIALS AND METHODS

Hepes, PMSF, DTT and all reagents for SDS-polyacrylamide gel electrophoresis were purchased from Sigma, Poole, England. GDP-β-S, GTP-γ-S and ATP were from Boehringer Mannheim, Lewes, England. Magnesium chloride, manganese chloride and sodium metavanadate were from BDH Chemicals, Poole, England. Porcine monocomponent insulin was a generous gift from Novo Research Institute, Copenhagen, Denmark. [^{32}P]Phosphate (PBS-11) was from Amersham, England and [γ-^{32}P]ATP was synthesized as described [20].

Membranes from human placenta were prepared and subsequently solubilised using Triton X-100 as described in some detail by Fujita-Yamaguchi et al. [21], except that 50 mM Hepes, pH 7.6, was used in place of the 50 mM Tris buffer. Furthermore, at the solubilisation step, the protease inhibitors pepstatin A, leupeptin and antipain were all added at 1 μg/ml as well as benzamidine (2.5 mM) and PMSF (0.1 mM). The insulin receptor kinase was purified to apparent homogeneity using a 'single-step' immunoadsorption technique employing a monoclonal antibody raised against a pure human insulin receptor preparation. This has been described in detail elsewhere [22,23]. Purification of G_i and G_o to apparent homogeneity was done as described in some detail by one of us [10]. For the phosphorylation assays 20 ng of insulin receptor, immobilised on an immunoadsorbent, were employed per assay (total volume 40 μl). This is equivalent to a concentration of 2 nM insulin receptor. The final Triton X-100 concentration in the assay was 0.1%. Unless stated otherwise, insulin (100 nM) was present.

The experiments performed involved a series of different pre-incubations prior to the actual phosphorylation assay.

2.1. *Pre-incubation mixture 1*

Pre-incubation mixture 1 was a total volume of 20 μl. It contained insulin receptor (4 nM) together with 24 nM $MgCl_2$, 4 mM $MnCl_2$, 0.2 mM $NaVO_3$ and 2 mM dithiothreitol in the presence or absence of 200 nM insulin. These were incubated for 15 min at 23°C prior to it being mixed together with the G_i/G_o preparation which had undergone its own pre-incubation (pre-incubation 2).

2.2. *Pre-incubation mixture 2*

Pre-incubation mixture 2 was a total volume of 10 μl and containing the G_i/G_o preparation at concentrations defined in the text together with either low (5 mM) or high (44 mM) $MgCl_2$ concentrations in the presence or absence of guanine nucleotide analogues (0.1 mM). The incubation of these components was done for 10 min at 32°C prior to its use.

2.3. *Pre-incubation mixture 3*

Pre-incubation mixture 3 consisted of taking material from pre-incubation 1 (20 μl) and mixing it together with the G_o/G_i mixture (10 μl) for pre-incubation 2 by vortexing prior to a further incubation of 10 min at a temperature of 23°C.

2.4. *Assay*

After the 10 min period observed in pre-incubation 3, this material (30 μl) was mixed with 10 μl of 0.4 mM [γ-^{32}P]ATP (2 μCi/nmol) which was then incubated for various time periods, up to 60 min, at 23°C. Reactions were terminated by addition of 3 × concentrated electrophoresis sample buffer (20 μl) containing 0.1875 M Tris, pH 6.8: 6% (w/v) SDS, 30% (v/v) glycerol, 15 mM EDTA, 300 mM DTT and 0.02% (w/v) bromophenol blue. After heating for 5 min at 100°C, samples were analysed by electrophoresis on SDS-polyacrylamide gels (150 × 150 × 1.5 mm), containing 17.5% (w/v) acrylamide [24]. Stained and dried gels were subjected to autoradiography. Results were quantified by scanning the autoradiographs using a Biorad gel scanner connected to an Olivetti M24 data processing system which allowed for background subtraction and image enhancement. In some instances quantification was done by excision of the appropriate regions from the gel and determination of ^{32}P by liquid scintillation counting.

Analysis of the phosphoamino acid content of ^{32}P-labelled proteins from SDS-polyacrylamide

gels was performed using either chromatography separation of phosphoamino acids or by determination of alkali-stable phosphate as described [25].

3. RESULTS AND DISCUSSION

Purification of the pertussis toxin-sensitive guanine nucleotide-regulatory protein activity from bovine brain yields a preparation which is now known to consist of two closely related G-proteins, G_i and G_o. These have apparently identical β-subunits of 35 kDa and closely related α-subunits of 40 and 39 kDa, respectively [10,11]. The preparation used in this study contained, as reported previously by us [10], only these two proteins. Because of their structural similarity, G_i and G_o are not easily separated or resolved on SDS-PAGE, although extended electrophoresis can effect the degree of resolution.

In the presence of GDP-β-S and at low [Mg^{2+}] (5 mM) both G_i and G_o are found in their intact, holomeric form [10,11,26,27]. Under such conditions the insulin receptor kinase elicited the phosphorylation (fig.1A) of the α-subunits of both

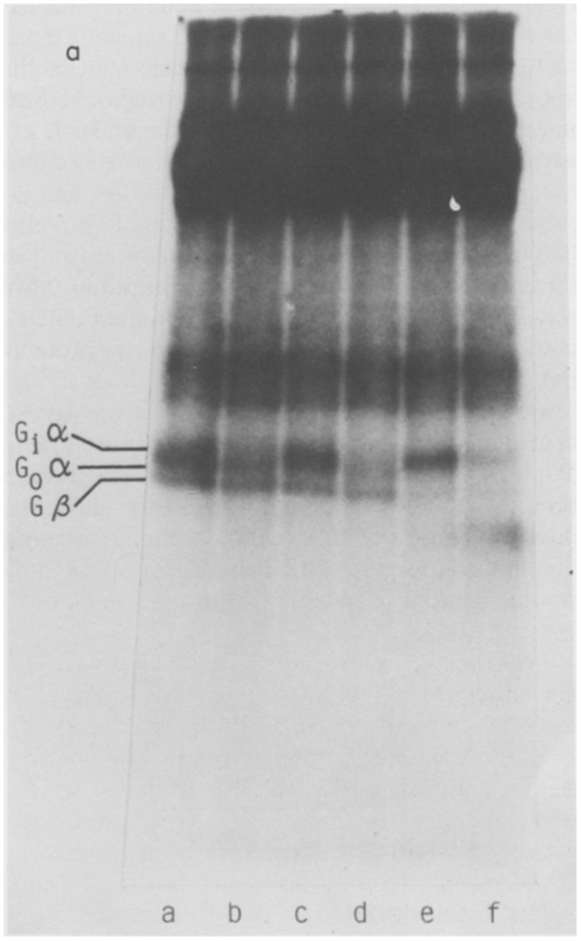

Fig.1. The phosphorylation of G_i and G_o by the insulin receptor tyrosyl kinase. (A) Shows a 15% SDS gel, aimed at resolving the α- and β-subunits G_i and G_o shown as indicated. The tracks were of experiments done at low (5 mM) [$MgCl_2$] (a,c,e) and high (44 mM) [$MgCl_2$] (b,d,f). Experiments were done with no added guanine nucleotides (a,b), with 110 μM GDP-β-S (c,d) and with 110 μM GTP-γ-S (e,f) present in pre-incubation 2 (see section 2). A typical experiment is shown using G_i/G_o at 0.3 μM. (B) Shows densitometric traces from experiments which highlight changes in the phosphorylation of the α- and β-subunits of G_i/G_o. Densitometric scans were performed over the molecular mass range encompassed by these subunits. These were performed as described in section 2 using a Biorad gel scanner under operating conditions which subtracted background exposures. Thus, during pre-incubation 2 the following conditions were employed with all guanine nucleotides present at 0.1 mM. Tracks: i, GDP-β-S in the presence of low (5 mM) $MgCl_2$, showing phosphorylation of the α-subunits of both G_i and G_o as well as their β-subunits; ii, GDP-β-S in the presence of high (44 mM) $MgCl_2$, showing the loss of phosphorylation of the α-subunit of G_i; iii, GTP-γ-S, at low (5 mM) $MgCl_2$, showing the selective loss of phosphorylation of the α-subunit of G_o; iv, GTP-γ-S at high (44 mM) $MgCl_2$ concentration, showing the loss of phosphorylation of all three components. Data from a typical experiment is shown using 15% gels.

G_i and G_o, as well as their β-subunits. This is shown in the autoradiograph of a typical experiment (fig.1A) and by a densitometric scan of the area of the gel where the β- and two α-subunits migrate (fig.1B). The quantification of such experiments is shown in fig.2. Furthermore, we also observed the phosphorylation of the α- and β-subunits of both G_i and G_o when these G-proteins had been pre-treated (figs 1,2) with low [Mg^{2+}] in the absence of GDP-β-S. Such conditions have also been shown to establish the holomeric, GDP-bound state of both of these G-proteins [10,11,26,27].

However, when full dissociation of these two G-proteins was achieved using GTP-γ-S at elevated [Mg^{2+}] (44 mM; see [26,27]), then the insulin receptor kinase failed (figs 1,2) to elicit the phosphorylation of the β- and α-subunits of both of these G-proteins. This demonstrates that only when G_i and G_o are in the holomeric form do their α- and β-subunits provide a substrate for the insulin receptor kinase. Such an action is reminiscent of the action of pertussis toxin which only elicits the ADP-ribosylation of the α-subunits of G_i and G_o when they are in their intact holomeric state [28,29].

G-proteins, including G_i and G_o, exhibit rather different affinities for Mg^{2+} and various guanine nucleotides and their analogues [26]. Indeed, the binding of GTP-γ-S to both G_i and G_o has been shown to be affected dramatically [26] by changes in the concentration of Mg^{2+}. Here we demonstrate that pretreating the bovine brain G_i/G_o preparation with high concentrations (44 mM) of Mg^{2+} alone, sufficed to reduce the ability of the insulin receptor tyrosyl kinase to phosphorylate the α-subunit of G_i (figs 1,2). This might be explained by the observation [26] that G_i

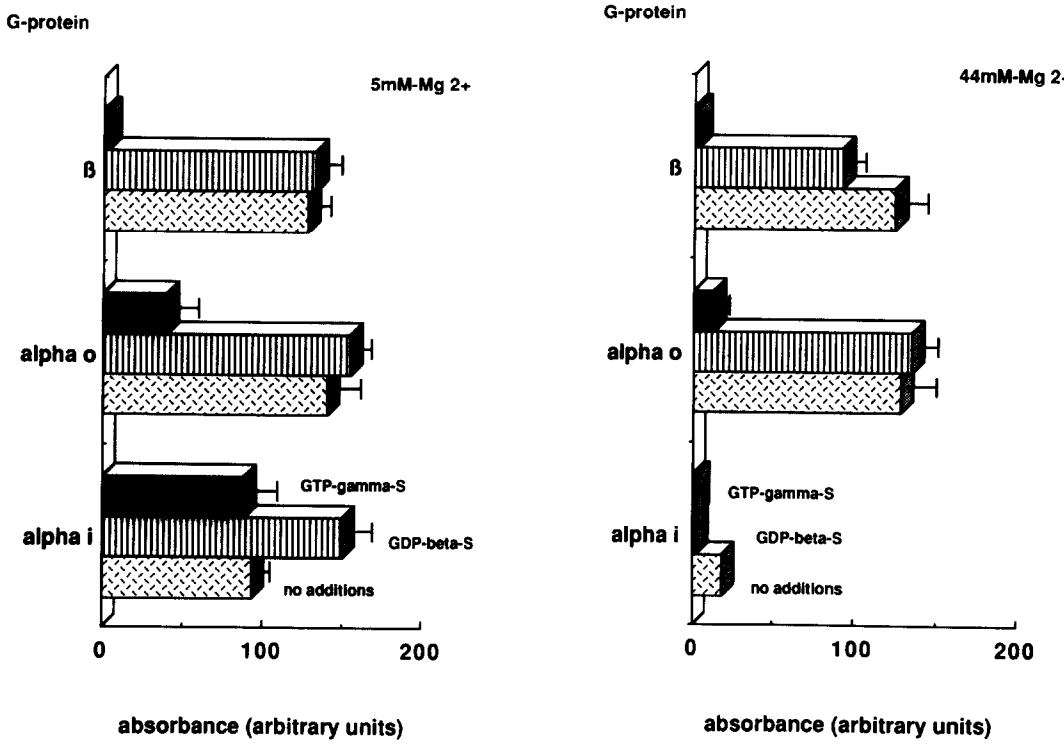

Fig.2. Effect of changes in $MgCl_2$ concentration and of guanine nucleotide analogues on the ability of the insulin receptor tyrosyl kinase to phosphorylate the α- and β-subunits of G_i and G_o. Histograms showing collected data of labelling from three experiments. These were carried out at both low (5 mM) and high (44 mM) $MgCl_2$ concentrations present in pre-incubation 2. Such experiments were performed in either the absence (flecked bars) of added guanine nucleotides or the presence of 100 μM GTP-γ-S (solid bars) or 110 μM GDP-β-S (vertical hatched bars). Labelling of the β- and α-subunits of G_i and G_o is shown. Errors are given as $\pm$ SD.

has a 10-fold greater affinity for Mg^{2+} than has G_o. In contrast to this, however, we observed that GTP-γ-S (100 μM), in the presence of low (5 mM) Mg^{2+} concentrations in the pre-incubation, sufficed to decrease the phosphorylation of the α-subunit of G_o but not that of the α-subunit of G_i (figs 1,2). This action would be consistent with the observation [26] that at low [Mg^{2+}], GTP-γ-S binds much more rapidly to the α-subunit of holomeric G_o than it does to that of G_i. It thus promotes the preferential dissociation of G_o, hence preventing its phosphorylation selectively. Thus by pre-treating the G_i/G_o preparation with appropriate concentrations of Mg^{2+} and with GTP-γ-S, we can differentiate between actions of the insulin receptor tyrosyl kinase in eliciting the phosphorylation of the α-subunits of G_i and G_o.

Phosphorylation of the β-subunit of the G_i/G_o preparation was little affected by the presence of either GDP-β-S or at high [Mg^{2+}] (fig.2). However, the presence of GTP-γ-S caused a dramatic decrease in the phosphorylation of this subunit. Indeed, this reduction in its ability to be phosphorylated was even more pronounced than was seen for the reduction in phosphorylation of the α-subunit of G_o. It is known, however, that GTP analogues cause the slow dissociation of G-proteins after inducing an appropriate conformational change [7]. It may be that it is the conformational change elicited by the initial binding of GTP-γ-S and not the subsequent dissociation of the G-protein which suffices to prevent the phosphorylation of β-subunits associated with the G_i/G_o preparation.

Distinguishing between actions of the kinase on G_i and G_o became difficult under conditions where the extent of phosphorylation was small, as was seen when using either low substrate concentrations or when incubation times were short. Therefore, under such conditions (fig.3) we measured the combined phosphorylation of the α-subunits of G_i and G_o, in the absence of added guanine nucleotides and at low [Mg^{2+}] (5 mM) where both proteins are holomers and subject to phosphorylation. In the absence of the G_i/G_o preparation no bands of phosphorylated material were found migrating to positions corresponding to their subunit molecular masses (fig.3A). We did note, however, evidence of a phosphorylated band migrating near the dye front with an apparent molecular mass of 8 kDa. This was only seen in tracks where G_i/G_o was present in the phosphorylation mixture. As such it may be the γ-subunit associated with both G_i and G_o [7], which could also be phosphorylated. This, however, was not pursued in this study. As before, linear time courses for phosphorylation were observed (fig.3B,C) and rates of phosphorylation were found to be proportional to receptor concentration (not shown). In these experiments we noted that insulin (100 nM), increased the rate of phosphorylation by some 59 ± 6% (see fig.3B,C). The relatively small effect of insulin, compared to that seen when insulin receptors are purified by other procedures can be attributed to a high 'basal' kinase activity seen in the absence of insulin. This was due to both the stimulation of the receptor by the antibody on the immunoadsorbent, which acts as a partial agonist [30], and also the autoactivation of the receptor as a result of autophosphorylation which occurred during the experiment, as has been shown by others [31].

The rate of phosphorylation of the G_i/G_o mixture was of the order of that observed with casein but less than that seen with histone H2B when similar concentrations (0.4 μM) of these substrates were employed. Thus, with the G_i/G_o mixture the rate was 1 nmol of phosphate incorporated/min per mg receptor protein; with casein it was 0.4 nmol/min per mg receptor protein and with histone 3 nmol of phosphate incorporated/min per mg receptor protein. The activity of the purified receptor towards histone and casein was of the same order as that reported by others [32,33]. Thus, 0.15 mol phosphate was incorporated per mol of the G_i/G_o over the period of assay (fig.3). However, incorporation of phosphate was still proceeding at a linear fashion even at 60 min. The low incorporation achieved here undoubtedly reflects the fact that assays were done under far from optimal conditions. Indeed, such conditions are necessary in order to achieve linear rates of phosphorylation for kinetic analysis. Thus the concentrations of G_i and G_o were well below their K_m value. Furthermore assays were performed at a below physiological temperature and the interacting components which are normally membrane-bound were used here in free solution rather than confined to diffusion in the two dimensions of a membrane lipid bilayer. We also noted that when

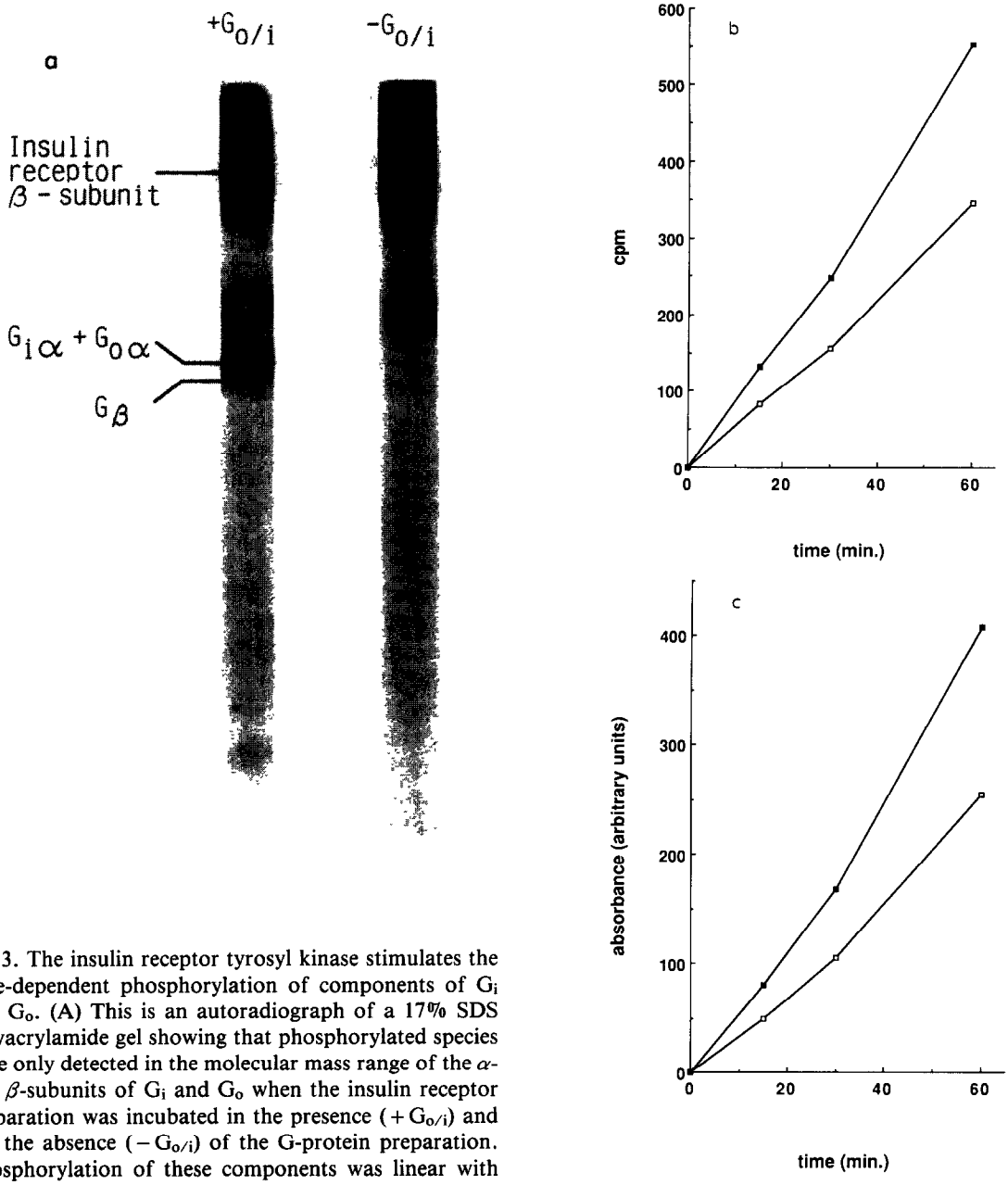

Fig.3. The insulin receptor tyrosyl kinase stimulates the time-dependent phosphorylation of components of G_i and G_o. (A) This is an autoradiograph of a 17% SDS polyacrylamide gel showing that phosphorylated species were only detected in the molecular mass range of the α- and β-subunits of G_i and G_o when the insulin receptor preparation was incubated in the presence ($+G_{o/i}$) and not the absence ($-G_{o/i}$) of the G-protein preparation. Phosphorylation of these components was linear with respect to time as assessed by both scintillation counting of excised (cpm) radioactive bands (B) and densitometric (absorbance) analysis (C). The phosphorylation of the subunits was followed in both the presence (■) and absence (□) of 100 nM insulin. These figures show that linear rates were found whether densitometric scanning or counting of excised bands was used to assess rates of phosphorylation. Data shown here is for the combined α-subunits. A typical experiment of one performed at least 6 times is shown here.

such experiments were performed at 4°C, rather than 23°C, the activity of the receptor kinase fell 10-fold and under these conditions, the rate of G_i/G_o phosphorylation was undetectable, although a low rate of histone phosphorylation was observed [34]. The K_m for phosphorylation of the G_i/G_o mixture by the insulin receptor kinase was estimated to be 31 μM in the absence of insulin

and this fell to a value of 19 μM in its presence. These compare with values of around 10–100 μM for histones, 10–20 μM for casein, 5–10 μM for Glu 4:Tyr 1, and 2–4 mM for angiotensin and the src-related peptide [32,33].

Analysis of the phosphoamino acid associated with the receptor β-subunit and with G_i/G_o showed it to be phosphotyrosine under all the various conditions of exposure to the G_i/G_o mixture and guanine nucleotides investigated above. Phosphorylation of the components of the G_i/G_o mixture by the insulin receptor tyrosyl kinase was stable to treatment with high concentrations of alkali and judged as described by others [25] to be essentially phosphotyrosine on that basis. However, levels of phosphoserine/phosphothreonine which were less than 20% of the total could not have been detected by us. We also noted that the presence of G_i and G_o, together with the various pre-treatments, did not affect the extent of autophosphorylation of the insulin receptor β-subunit (<5% change).

These experiments demonstrate that the purified insulin receptor will cause the phosphorylation of the β- and α-subunits of both G_i and G_o. However, such phosphorylation only occurred when the proteins were in their holomeric state. This indicates that particular conformational requirements have to be satisfied for this event to occur. Indeed, this action bears analogy with that of pertussis toxin which only elicits the ADP-ribosylation of the α-subunits of G_i and G_o when they are in their holomeric state. It remains to be seen as to whether this action of the insulin receptor tyrosyl kinase can prevent the dissociation and functioning of these two G-proteins as does modification by both pertussis toxin [7,8,10,11] and protein kinase C on G_i [34]. It also needs to be investigated as to whether such a modification can occur, in situ, in either intact cells or isolated plasma membranes. Indeed under such conditions the concentration of proteins, diffusing in the 2-D of the bilayer will be proportionally higher than in free solution and their collision frequency will be increased markedly. Thus, one might expect the observed rate of reaction and hence phosphorylation to be increased by at least 100-fold. We would like to suggest that our results provide support for the notion that the insulin receptor can interact with and perhaps modify signal transduction through the G-protein system. Whether phosphorylation effects are confined to G_i and G_o or extend to other G-proteins, including the putative G_{ins} [13], remains to be determined.

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